Vitis **41** (3), 115–121 (2002)

Evidence for the involvement of cysteine proteases in the regulation of methyl jasmonate-induced cell death in grapevine

V. Repka

Laboratory of Molecular Biology and Virology, Research Institute of Viticulture and Enology (CRIVE), Bratislava, Slovakia

Summary

A new system to study programmed cell death (PCD) in plants is described. Grapevine (*Vitis vinifera* L., cv. Limberger) leaves and suspension cells were induced to undergo a form of cell death that mimics the hypersensitive response (HR) by treatment with a lipid-derived molecule, methyl jasmonate (MeJA). This chemical-induced cell death was accompanied by the characteristic features of apoptosis in animal and plant cells, such as typical changes in nuclear morphology, the fragmentation of the nucleus and protoplast collapse. Local and ectopic treatment of grapevine leaves with phenylmethylsulfonyl fluoride (PMSF), leupeptin, and especially with a specific inhibitor of cysteine proteases, E-64, inhibited MeJA-induced cysteine protease activity and blocked PCD triggered by 50 μM MeJA. These results indicate that proteolysis plays a crucial role in MeJA-induced apoptosis and that this type of PCD can be regulated by activity poised between the cysteine protease and the cysteine protease inhibitor.

K e y w o r d s : *Vitis vinifera* L., Limberger, caspases, apoptosis, protease inhibitors.

Abbreviations: AEBSF, 4-[2-aminoethyl]-benzesulfonylfluoride; DIC, differential interference contrast; DMSO, dimethyl sulfoxide; E-64, *trans*-(epoxysuccinyl)-L-leucylamino-(4-guanidino) butane; NIA, necrosis inducing activity; PMFS, phenylmethylsulfonyl fluoride.

Introduction

Programmed cell death (PCD) is an intrinsic part of the life cycle of all multicellular organisms studied so far, including both animals and plants (Ellis *et al*. 1991; Greenberg 1996; Pennell and Lamb 1997; Green 1998). Studies on animal systems have shown that the execution of PCD or apoptosis is controlled by a multistep signaling pathway (McConkey and Orrenius 1994; Stewart 1994). In plants, PCD is an integral part of normal development, including embryogenesis, floral organ abortion, root cap sloughing, senescence, and the development of gametophytes and vascular tissue (Jones and Dangl 1996; Buchanan-Wollaston 1997; Fukuda 1997; Pennel and Lamb 1997; Mittler 1998; Young and Gallie 1999; Xu and Hanson 2000). Recently, some apoptotic features have been detected during plant cell death associated with the hypersensitive response to pathogens (Ryerson and Heath 1996; Mittler and Lam 1997), phytotoxins (Wang *et al*. 1996; Navarre and Wolpert 1999; Asai *et al*. 2000) and environmental stress (Lamb and Dixon 1997; McCabe *et al*. 1997; Danon and Gallois 1998).

Although a detailed understanding of how plant cells die is still largely unknown, recent studies have shown that the apoptotic pathways are conserved over a wide range of phyla. Specifically, the morphological hallmarks of apoptosis include cytoplasmic shrinkage, nuclear condensation, and membrane blebbing (Earnshaw 1995; Martins and Earnshaw 1997); the biochemical events involve calcium influx, exposure of phosphatidylserine, and DNA fragmentation known as DNA laddering (Stewart 1994; Wang *et al*. 1996; O'Brien *et al*. 1998). At the molecular level, apoptotic stimuli deactivate the suppression of PCD by anti-apoptotic molecules that unleash the protease activities of a subset of cysteine (Cys) proteases, the caspases (*c*ysteine *aspa*rta*ses*). Activation of dormant caspases constitutes the critical point in the PCD pathway. However, so far, no caspase genes or caspase enzymes have been isolated from plants. Recently, such activity has been reported in tobacco mosaic virus-infected tobacco plants when a short peptide AMC (Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin) substrate was used (Del Pozo and Lam 1998). Furthermore, caspase-specific peptide inhibitors were found to be potent inhibitors of the chemical-induced apoptosis in tomato cells (De Jong *et al*. 2000), indicating that, as in animal systems, caspase-like proteases are involved in the apoptotic cell death pathway in plants. Other compounds that were effective in reducing the degree of PCD in plants, namely leupeptin, PMSF, and AEBSF, all have inhibitory activity against Cys proteases (Alonso *et al*. 1996).

In plants and other organisms, protease activity can be regulated at different levels: by transcription/translation, by post-translational processing, and by specific protease inhibitors (Bode and Huber 1992). Previous studies indicated that jasmonate-induced cell death in grapevine is augmented by the inhibition of some aminopeptidases with bestatin (Repka 2002 b). Therefore, to extend our initial characterization of the molecular mechanisms involved in jasmonate-

Correspondence to: Dr. V. Repka, Laboratory of Molecular Biology and Virology, Research Institute of Viticulture and Enology (CRIVE), Matúškova 25, SK-833 11 Bratislava, Slovakia. Fax: +421-2-5477-54 36. E-mail: vrepka@vuvv.sk

induced HR-like cell death it was examined whether exogenous cysteine protease inhibitors may function in modifying this type of PCD.

Material and Methods

Plant material: Grapevine (*Vitis vinifera* L. cv. Limberger) plants were grown in a growth chamber at 28±1 °C (RH 60 %) with a 14 h light period (30 μmol m^{-2} s^{-1}). Intact two-month-old plants or excised leaves were used for all experiments. A suspension cell culture was established and maintained as described by REPKA *et al.* (2000). Cell cultures were used for the experiments 4 d after subculturing.

Treatment of plants, excised leaves or cell suspensions with MeJA and protease inhibitors: Methyl jasmonate (50 μM MeJA, Duchefa, Haarlem, The Netherlands) was prepared from a stock solution in absolute ethanol (0.1 % final concentration). MeJA was applied at the concentrations indicated as 0.01 ml droplets on intact plants or on excised leaves (three drops per leaf). Alternatively, plant cuttings in 2.5 ml of water were exposed to MeJA vapour in air-tight Magenta containers (Magenta Corp., Chicago, USA) containing cotton-tipped wooden dowels to which had been applied 0.01 ml of dilutions of MeJA in 0.1 % ethanol or 0.1 % ethanol alone as a control. The cotton tip was placed about 4 cm from the plant leaves. The chambers were incubated in constant light (30 μmol m^{-2} s^{-1}) at 25 °C for 8 h.

For experiments on effects of different protease inhibitors on MeJA-inducible hypersensitive response, grapevine leaves were treated with the inhibitors (EDTA, 1 mM; bestatin, 5 μg ml^{-1}; pepstatin, 5 μg ml^{-1}; leupeptin, 5 μg ml^{-1}; PMSF, 5 μg ml^{-1}; E-64, 5 μg ml^{-1}) for 24 h. Except EDTA, which was dissolved in sterile water, other inhibitors (Amresco, Solon, USA) were dissolved in DMSO. Care was taken to keep the final DMSO concentration less than 0.1 % (v/v), at which concentration it does not exhibit elicitor activity or inhibit elicitation responses. Treatment of test cells with MeJA and protease inhibitors was performed in 24-well microtiter plates (Nunc AS, Copenhagen, Denmark) with 5 ml aliquots of cell culture per well. The cells were treated with the respective inhibitor for 24 h prior to treatment with 50 μM MeJA and collected after the following 24 h by filtration for protein extraction.

Biological assays: Necrosis-inducing activity (NIA) of MeJA and various protease inhibitors was assayed either on 2-month-old grapevine plants grown in a growth chamber under the conditions described above or on excised grapevine leaves cut at the base of their petioles and maintained in 2 ml Eppendorf tubes containing tap water under constant light (30 μmol m^{-2} s^{-1}) at 25 °C. Routinely, 10 μl drops of MeJA (50 μM in 10 % (v/v) ethanol) or protease inhibitors, 10 % ethanol and 0.1 % DMSO alone, and sterile distilled water were applied on intact or excised leaves.

Following MeJA treatments, leaves with necroses were laid flat on dark screen and photographed on slide film beside a reference ruler. Slides were projected onto a digitizing tablet (model 1212, Kurta, Banska Bystrica, Slovakia), and necroses were measured directly using SigmaScan software (Jandel Scientific, San Rafael, USA). Unless otherwise indicated, NIA inhibition data represent results from two or three independent replicates containing 3 - 5 leaves per treatment.

Measurement of cell death: To determine changes in plant cell viability, cell cultures were incubated for 15 min with 0.05 % Evans blue (Sigma, Deisenhofen, Germany) and then washed extensively to remove excess and unbound dye. Dye bound to dead cells was solubilized in 50 % methanol with 1 % SDS for 30 min at 50 °C and quantified by absorbance according to REPKA (2001). Data are means of two or more replicates.

Preparation of protein extracts and in-gel protease assay: To prepare extracts from control and treated plant material, leaves or cells were mixed with 3 volumes (w/v) of ice-cold extraction buffer (20 mM Tris-HCl, pH 7.8, 20 mM NaCl, 1 mM DTT, 1 mM EDTA and 0.6 % PVP) and homogenized using a prechilled minimortar and pestle (Kontes, Vineland, USA). Homogenates were centrifuged at 20,000 *g* for 10 min at 4 °C, the clear supernatant was concentrated using a Vivapore 5 solvent absorption miniconcentrator (Vivascience, Lincoln, UK) and stored at -20 °C until further use. For in-gel protease assay (LAEMMLI 1970) loading buffer was added to the supernatant, and samples were incubated at 37 °C for 5 min before loading on a 10 % SDS-polyacrylamide gel containing 0.1 % gelatin. Renaturation was done by two washes with 10 mM Tris, pH 7.5, and 0.25 % Triton X-100 for 45 min each. The gel was incubated overnight at 30 °C and stained with Coomassie Brilliant Blue R-250. Active proteases digested the gelatin and appeared as white bands. After staining the gels were scanned in a ScanJet 3200C transparency scanner (Hewlett-Packard, Palo Alto, USA) in the RGB mode, converted to a greyscale mode, and inverted with Adobe PhotoShop (Adobe Systems, San José, USA).

Microscopy: Nuclei of treated and control cells were stained by incubation of cells with the fluorescent probe DAPI (Molecular Probes, Eugene, USA) at 0.5 μg ml^{-1} in 0.1 M Tris-HCl, pH 9.0 for 10 min. Stained cells were examined using excitation light at 361 nm. Fluorescence and concurrent differential interference contrast (DIC) images were recorded in optical sections and time series modes with Olympus AX-70 microscope (Olympus, Tokyo, Japan). Images were captured using a cooled charge-coupled device camera Progressive 3 (Sony, Tokyo, Japan), transformed into three-dimensional reconstructions with a surface shadowing algorithm in the Olympus software; orthogonal slices were produced by using Metamorph (Universal Imaging, West Chester, USA) and processed with Adobe PhotoShop v.5.0 (Adobe Systems, San José, USA). At least 50 cells were examined for each experiment and three independent experiments were performed for each treatment.

Results

Cysteine protease inhibitors prevent cell death induced by MeJA: To assess the role of specific types of proteases in grapevine cell death elicited by MeJA, in a first step 6 different protease inhibitors were injected into grapevine leaves 0 or 24 h prior to treat-

ment with 50 μM MeJA. Experimental treatments were performed on grapevine leaves basically according to the scheme shown in Fig. 1 A. As expected, visual determination of the extent of necroses induced 24 h after the onset of treatment confirmed that none of the inhibitors significantly inhibited MeJA-induced cell death while they were applicated simultaneously with MeJA (Fig. 1 B). In contrast, a distinct pattern was observed when the grapevine leaves were injected with 50 μM MeJA after 24 h pretreatment with each inhibitor. In this experiment, PMSF, leupeptin and E-64 reproducibly caused a strong inhibition response towards MeJA-induced cell death (Fig. 1 C). However, pretreatment of grapevine leaves with EDTA (an inhibitor of metalloproteinases), bestatin (an inhibitor of some aminopeptidases) or pepstatin (an inhibitor of acidic aspartic proteases) did not affect the ability of MeJA to induce cell death. From the absence of necrosis-inducing activity (NIA) in the part of the leaf that was injected by the sole protease inhibitor and the fact that only a subset of the tested protease inhibitors effectively suppressed MeJA-induced cell death it can be argued that the implementation of this type of PCD requires the degradation of specific polypeptides.

A specific cysteine protease is required for MeJA–induced cell death: To examine the requirement for cysteine protease during jasmonate-induced cell death, we first investigated the necrotic response following injection of 50 μM MeJA into grapevine leaves pretreated with different protease inhibitors for 24 h. As shown in Fig. 2 A, 24 h after the onset of treatment MeJA produced localized cell death with a "signature" that reflects the substrate specificity of the inhibitors used. To perform an analysis of the efficiency of the respective protease inhibitors to inhibit necrosis inducing activity (NIA) of MeJA, the shape of necroses produced by MeJA on leaves pretreated with inhibitors was optically-traced using a sophisticated software (Fig. 2 B) and quantified (Fig. 2 C). E-64 (5 μg ml^{-1}), a specific inhibitor of cysteine proteases was shown to be the most potent inhibitor of MeJA-induced cell death. In this case, the shape of necroses was considerably reduced and the rate of NIA inhibition reached a value close to 100 % (97 %). Also PMSF (5 μg ml^{-1}) and leupeptin (5 μg ml^{-1}), the compounds that both have some inhibitory activity against Cys proteases, were almost to the same extent effective in reducing the degree of MeJA-induced cell death. Both inhibited the NIA of MeJA to 50 % when compared to the other three inhibitors (EDTA, bestatin, pepstatin) but they exhibited about 5-fold lower effectiveness than the specific inhibitor E-64.

In a similar experiment, excised leaves were either locally-injected or ectopically-treated with a range of concentrations of the very potent cysteine protease inhibitor E-64 (Fig. 3). Typically, localized pretreatment of leaves with E-64 substantially blocked MeJA-induced cell death in a concentration-dependent manner (Fig. 3 A, B). Moreover, the result of this experiment also demonstrates that inhibition of MeJA-dependent HR-like cell death was maximal at 40 μg ml^{-1}. In contrast, ectopic application of E-64 to grapevine had a more profound effect and completely blocked the formation of HR-like lesions at concentration higher than 5 μg ml^{-1} (Fig. 3 C, D).

Cysteine protease inhibitor E-64 blocks both, MeJA–induced protease activity and cell death: To obtain further evidence for the involvement of the specific cysteine protease in MeJA-triggered PCD pathway, cellular proteins were separated by SDS-PAGE and used for the in-gel protease activity assay. The results shown in Fig. 4 A (lane 3) reveal ladder-like bands that appear as early as 30 min after induc-

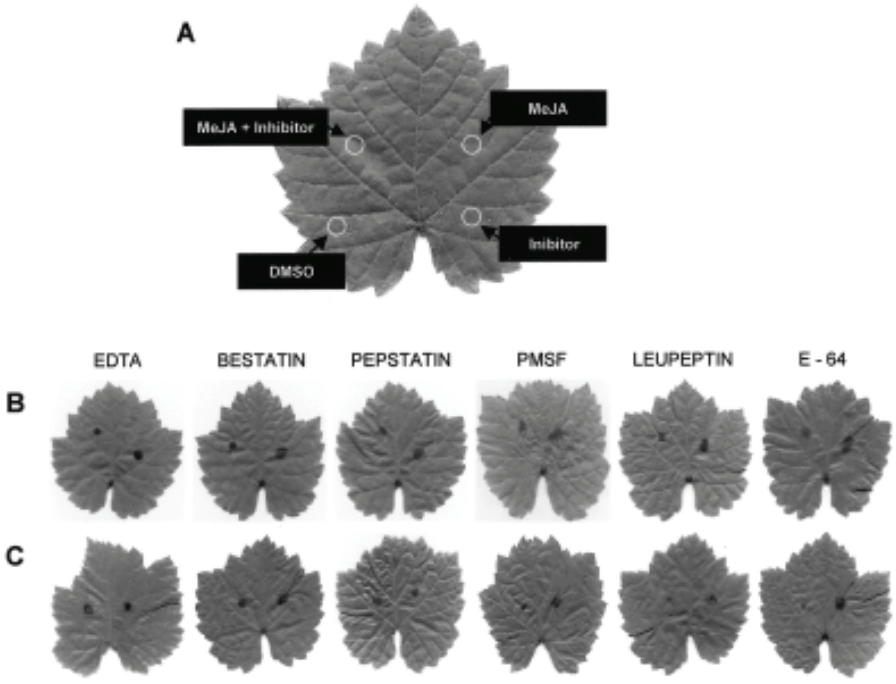

Fig. 1: Inhibition of MeJA-induced necrosis inducing activity (NIA) after pretreatment with different protease inhibitors. **A**: Test solutions applied locally. **B**: Simultaneous treatment with 50 μM MeJA and indicated protease inhibitors. **C**: Pretreatment with different protease inhibitors for 24 h prior to challenge with 50 μM MeJA. Photo taken 2 h after treatment with MeJA.

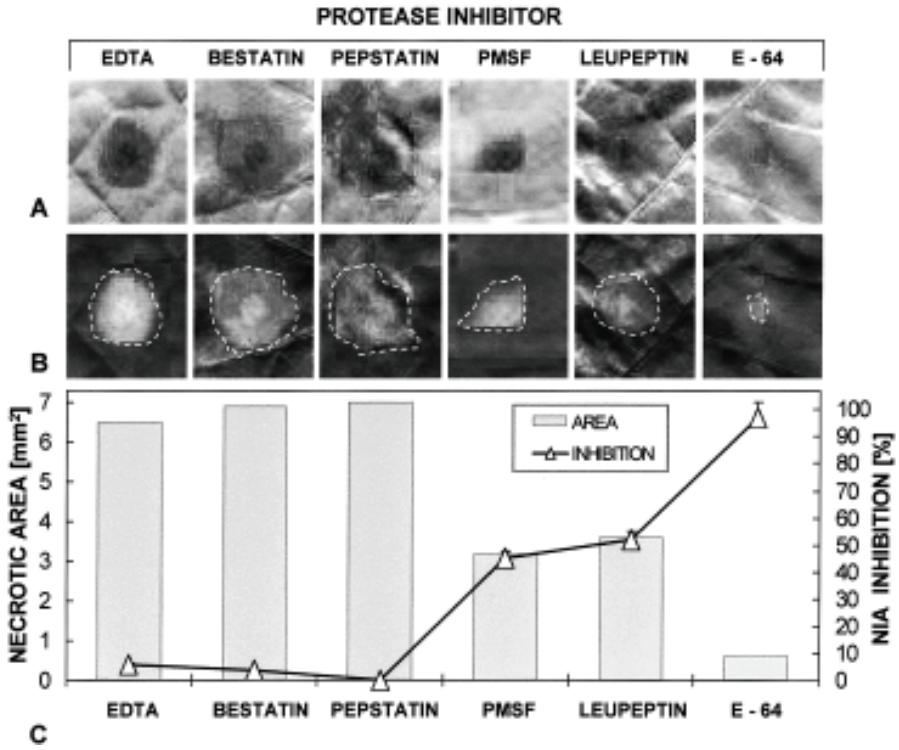

Fig. 2: Inhibition of MeJA-triggered necrosis inducing activity (NIA) in leaves ectopically-pretreated with different protease inhibitors. **A**: Excised leaves supplied with the respective protease inhibitors for 6 h, then challenged with 50 μM MeJA; NIA evaluation 2 h later. **B**: Part of leaves bearing necroses under UV light were optically traced to calculate surface of the necrotic area. **C**: Correlation between the MeJA-induced necrotic response and NIA inhibition by various protease inhibitors. Bars indicate ± SD.

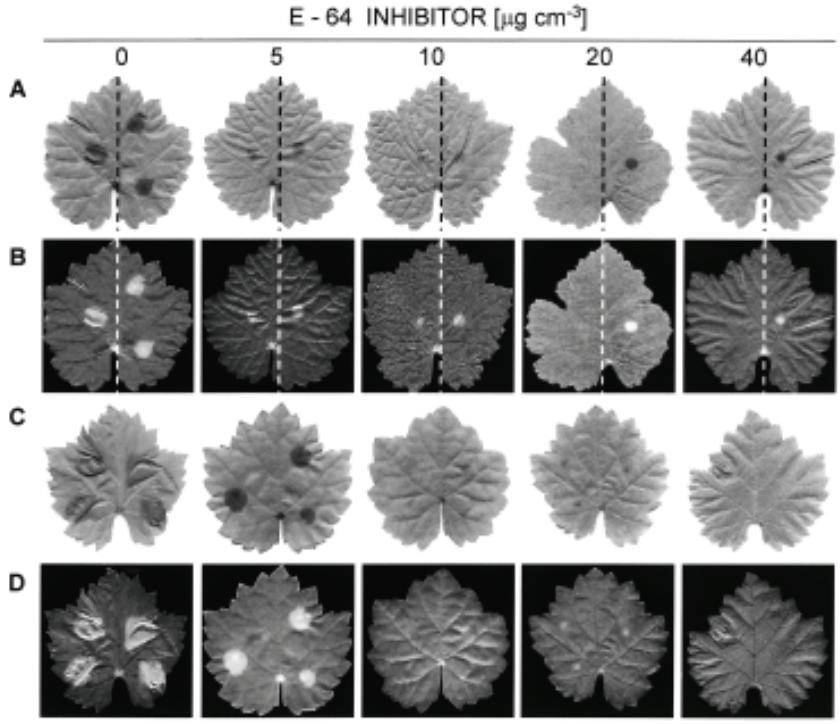

Fig. 3: Dose-response for the inhibition of necrosis inducing activity (NIA) by E-64. **A**: Excised leaves locally pretreated with various concentrations of E-64 for 2 h, then locally inoculated with 50 μM MeJA at the same sites. Inhibitor-pretreated (left), 50 μM MeJA alone (right). **B**: Leaves as in **A** but viewed under UV light to increase the visualization of NIA. **C**: Excised grapevine leaves ectopically pretreated with different amounts of E-64 prior to challenge with 50 μM MeJA. **D**: Leaves as in **C** but viewed under UV light. All photos taken 2 h after stimulation with MeJA.

tion of PCD by MeJA. Proteolytic activity could be traced to cytosolic fraction, and no additional bands were detected after a longer incubation up to 6 h after stimulation (data not shown). Preincubation of grapevine cell suspensions with the specific cysteine protease inhibitor E-64 (20 μg ml^{-1}) for 24 h completely inhibited MeJA-induced proteolytic activ-

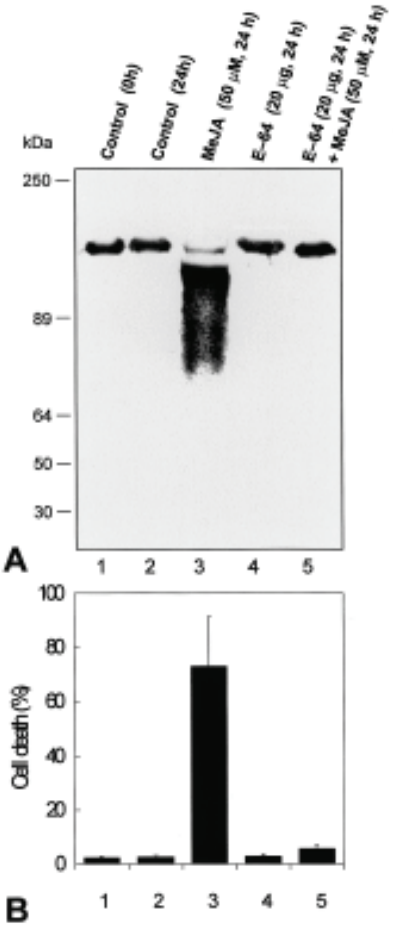

Fig. 4: Stimulation of proteolytic activity and cell death by MeJA and their inhibition by a specific inhibitor of cysteine proteases. **A**: Suspension-cultured grapevine cells treated with 50 µM MeJA, E-64 (20 µg ml^{-1}) and water (control), or pretreated with E-64 (20 µg ml^{-1}) and then challenged with 50 µM MeJA. **B**: Aliquots of cell suspensions treated as in **A** assayed for cell death. Bars indicate ± SD.

ity (Fig. 4 A, lane 5). The addition of E-64 alone at the same concentration as indicated above did not affect the proteolysis (Fig. 4 A, lane 4). The same was true for both control treatments with sterile distilled water (0 and 24 h, Fig. 4 A, lane 1 and 2, respectively).

These results raise the question whether the observed proteolytic activity is correlated with MeJA-induced PCD or whether the entire cellular machinery for the execution of PCD was present before application of the octadecanoid stimulus. This analysis revealed that, within our experimental limits, addition of the cysteine protease inhibitor E-64 to grapevine cell suspensions led to an effective block in MeJA-induced PCD (Fig. 4 B, lane 5), implicating the cysteine protease in the MeJA-triggered pathway that leads to apoptosis. Overall inhibition of jasmonate-induced PCD by inhibitor E-64 leads to a value about 10-fold lower than that observed after addition of 50 µM MeJA as the sole agent (Fig. 4 B, lane 3). On the other hand, the addition of E-64 alone to cell cultures exerts no visible outcome on the overall development of PCD (Fig. 4 B, lane 4). Likewise for proteolytic activity assays, treatment with sterile distilled water for either 0 or 24 h did not substantially increase the fraction of cells undergoing PCD (Fig. 4 B, lanes 1 and 2).

Pretreatment with an exogenous cysteine protease inhibitor represses MeJA–triggered morphological hallmarks of PCD in grapevine cells: To find out whether the jasmonate-induced cell death in grapevine cells exhibited any of the characteristic hallmarks associated with PCD in higher plants, the treated cells were microscopically analysed for the presence of some morphological features, namely internucleosomal fragmentation of DNA and condensation of the protoplast away from the cell wall. Microscopic examination of MeJA-treated cells (Fig. 5 A, lane 2, top panel) revealed that treatment with 50 µM MeJA triggered the condensation of the protoplast (arrow heads) away from the cell wall of the dead cells. Moreover, jasmonate treatment consistently induced shrinkage of the nucleus (Fig. 5 A, lane 2, middle panel) and the chromatin condensation, as visualized by the occurrence of intranuclear, punctuate pattern in DAPI-stained nuclei (Fig. 5 A, lane 2, bottom panel). The rate of nuclear fragmentation was further confirmed by laser scanning topography and is represented as a 3D-histogram (Fig. 5 B, graph 2). By contrast, treatment of cells with E-64 (20 µg ml^{-1}) prior to stimulation with MeJA repressed both apoptosis-related events, *i.e.* protoplast col-

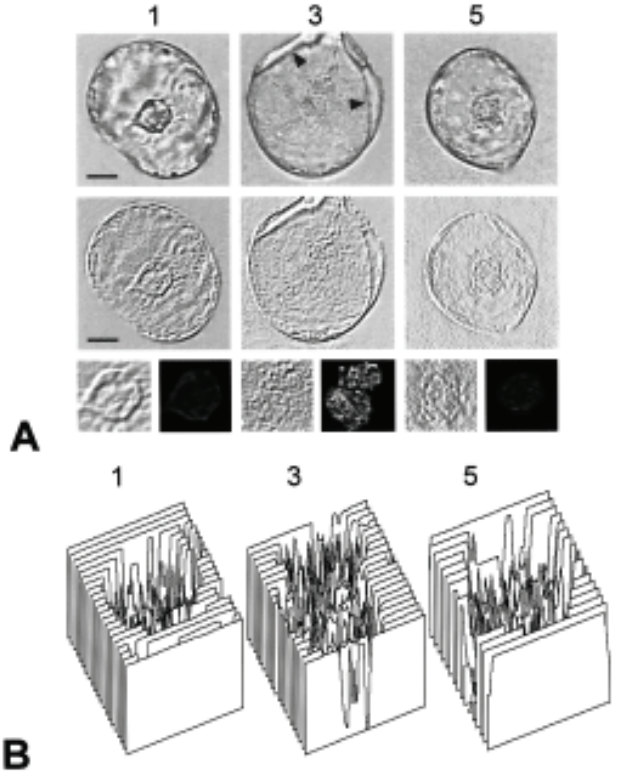

Fig. 5: Apoptotic changes in cells treated with MeJA and E-64. **A**: Cells taken from treatments as indicated in Fig. 4 A. Control cell (column 1) with normal mesh-like structure of chromatin and protoplast integrity. Treatment with 50 µM MeJA (column 2) triggers both progressive nuclear fragmentation and protoplast collapse (arrow heads). Pretreatment with E-64 (column 3) blocks MeJA-triggered signs of apoptosis. Top panels: phase contrast view, middle panels: concurrent DIC, bottom panels: a magnified view of nucleus observed under DIC (left) or UV light after DAPI staining (right). Bars = 0.5 µm. **B**: Plots of nuclei in the cells treated as above indicating the rate of chromatin fragmentation, as measured by laser scanning topography.

lapse as well as the above-mentioned intranuclear punctuate structures were seldom observed (Fig. 5 A, lane 3). The absence of significant disorganisation of the nucleus was also confirmed by scanning topography (Fig. 5 B, graph 3 as compared to graph 1). Control cells did not show visible signs neither of protoplast condensation nor of nuclear fragmentation and remained alive throughout the entire experiment (Fig. 5 A, lane 1). Hence, these results clearly document that jasmonate-triggered cell death in grapevine cells is apoptotic in nature.

Discussion

In the reported work, we focused our investigations on the events that during treatment with methyl jasmonate lead to the HR-like cell death and on the requirement for specific protease in this process. Furthermore, a model system was established to reproducibly induce apoptosis in grapevine suspension cells.

Previous work has shown that in excised grapevine leaves or in cultured cells, the cell death process that mimics the HR is triggered by exogenous MeJA and could be specifically modulated by tunikamycin, an inhibitor of N-linked glycosylation, as well as with bestatin, an inhibitor of some aminopeptidases (REPKA and FISCHEROVÁ 2001; REPKA 2002 b). In this work, pre-treatment of grapevine leaves with 6 different protease inhibitors prior to stimulation with 50 μM MeJA revealed that only a small subset of inhibitors, especially those that either non-specifically (PMSF and leupeptin) or specifically (E-64) inhibit the Cys proteases substantially blocked MeJA-triggered HR-like cell death. In analogy with animal systems, different classes of proteases play a role in plant PCD. Several serine and cysteine proteases are associated with xylogenesis (YE and VARNER 1996; GROOVER and JONES 1999). Phytepsin, a barley vacuolar aspartic protease is involved in PCD of nucellar cells during degeneration (CHEN and FOOLAD 1997). Moreover, a matrix metalloproteinase gene, *Cs1-MMP*, is expressed at the boundary of senescence and PCD in cucumber (DELORME *et al*. 2000).

As demonstrated by in-gel protease assay as well as with *in vitro*-cultivated grapevine cells only E-64 specifically inhibited MeJA-stimulated cell death and supressed apoptotic-related events like nucleolar fragmentation and protoplast condensation both thought to be prominent hallmarks of plant apoptosis. Furthermore, in line with these findings is the observation that cysteinyl proteases are major executors of PCD in both plants and animals (MARTIN and GREEN 1995; COHEN 1997; SOLOMON *et al*. 1999; DE JONG *et al*. 2000). Mutations in the Cys protease gene *Ced-3* prevent normal cell death in nematodes. Conversely, overexpression of the specific cysteine protease genes causes cell death in many cell types and viral genes that inhibit specific cysteine proteases prevent apoptosis of host cells (XUE and HORWITZ 1995). In addition to examples given above, expression of some baculovirus anti-apoptotic proteins (IAP-inhibitor of apoptosis) that act as caspase inhibitors, in transgenic tobaccos conferred heritable resistance to several necrotrophic fungal pathogens. More interestingly, the transgenic tobacco plants also displayed resistance to a necrogenic virus (DICKMAN *et al*. 2001).

Our results suggest that plants have the ability to control PCD by inhibiting and/or stimulating cysteine proteases that regulate the expression of specific protease inhibitor genes. In this context, we hypothesize that MeJA may exert dual activity in regulating PCD in plants. First, it is the negative type of regulation of the PCD via activating the genes encoding protease inhibitors, *e.g.* type I and II from Solanaceae and trypsin inhibitor from Fabaceae (FARMER and RYAN 1990; FARMER *et al*. 1992). Interestingly, in plants, protease inhibitor genes also are subject to regulation by intracellular signaling molecules, such as jasmonates (FARMER *et al*. 1992; BOTELLA *et al.* 1996). Thus the purpose of PCD inhibition by jasmonate could be to downregulate the PCD response in cases in which PCD has no survival advantage, for example, during attack by herbivores. In this case, jasmonate shifts the balance toward "life" and in reality it may act to prevent excess cell death caused by the release of peroxides from cells crushed by grazing. Second, a positive regulation of PCD by activating a cascade of events leading to cell death and eventually to apoptosis. This may be a less conserved mechanism as the MeJA-triggered cell death has been demonstrated to occur only in woody plant species (REPKA 2000 a). Involvement of MeJA in PCD in plants, however, is not without precedent. Note that other lipid-derived compounds act as signals that regulate apoptosis in animals and plants. Most intriguing is the recent and novel involvement of ceramide-related compounds, *e.g.* sphinganine or Fumonisin B_1, in affecting apoptosis (HANNUN and OBEID 1995; STONE *et al*. 2000). Interestingly, Fumonisin B_1-induced cell death in *Arabidopsis* protoplast requires jasmonate-dependent signaling pathway (ASAI *et al*. 2000) but to date, there is no information available whether this type of cell death also requires proteolysis of specific proteins.

In summary, our data show the importance of plant cysteine proteases in the implementation of an apoptotic process, and offer new ways to investigate the mechanism by which jasmonate-triggered stimuli activate cell death. The question how a cysteine protease could be involved in the transduction of an apoptotic signal mediated by MeJA might be found by identifying interacting partners. A biochemical approach to establish which proteases are affected by the cysteine-specific inhibitor E-64 in jasmonate-induced PCD in grapevine suspension cells is currently undertaken.

Acknowledgements

Mrs. I. FISCHEROVÁ is gratefully acknowledged for her expert and dependable assistance. This research was supported in part by a grant of the Slovak Ministry of Agriculture (No. STP-27-19).

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Received April 27, 2002